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⑤④ **Genetic engineering of cotton plants and lines.**

⑤⑦ A method is disclosed to achieve genetic transformation of cotton plants and lines. Immature cotton tissues are genetically transformed in vitro, by Agrobacterium-mediated genetic transformation. The resultant cotton tissues are subjected to a selection agent or agents to screen for transformants. The transformed cultures are then induced to commence somatic embryogenesis. One possible regime for regenerating such somatic embryos into whole cotton plants is disclosed.

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Description

GENETIC ENGINEERING OF COTTON PLANTS AND LINES

Field of the Invention

5 The present invention relates to the general technology of plant genetic engineering, and is related, in particular, to a strategy for the transformation and regeneration of cotton (*Gossypium hirsutum* L.) plants using the *Agrobacterium* method of plant genetic transformation so as to create novel genetically transformed cotton plants and lines.

10 Background of the Invention

It is a general objective by many scientists working in the field of plant biotechnology to successfully genetically engineer plants of major crop varieties. While plant genetic engineering has been successfully demonstrated in several model plant species, often the model plant species, such as tobacco, carrot and petunia, are not the most economically important plant species for agricultural purposes. Accordingly, much effort has been directed toward the genetic engineering of the more agriculturally important plant species. By the term "genetic engineering" as used herein it is meant to describe the introduction of foreign, often chimeric, genes into one or more plant cells which can be regenerated into whole, sexually competent, viable plants which can be self-pollinated or cross-pollinated with other plants of the same species so that the foreign gene, carried in the germ line, can be inserted into or bred into agriculturally useful plant varieties.

20 The art of plant tissue culture has been an area of active research for many years but over the past five to ten years an intensified scientific effort has been made to develop regenerable plant tissue culture procedures for the important agricultural crops such as maize, wheat, rice, soybeans, and cotton.

Early publications on tissue culture of cotton dealt mainly with establishing the growing tissues from the plant under aseptic conditions *in vitro*. Davis, D.G. et al. *In Vitro* 9:395-398 (1974); Rani, A. and S.S. Bhojwani, *Plant Sci. Lett.* 7:163-169 (1976); and Price, H.J. et al., *Plant Sci. Lett.* 10:115-119 (1977). The methods detailed in these publications, however, did not provide the necessary framework needed to regenerate cells back into whole plants.

In the late 1970's, the development of somatic embryos, i.e., embryos derived from nongametic or somatic tissues, from the wild cotton species *G. klotzchianum* was reported. Price, H.J. and R.H. Smith, *Planta* 145:305-307 (1979). Unfortunately, there were two major problems those using the procedure of this report were unable to overcome. First, even after several more years of research, these investigators could not induce the somatic embryos to germinate, i.e., convert, to give rise to whole plants. Finer, J.J. and R.H. Smith, *Plant Cell Rep.* 3:41-43, 1984. Secondly, the same technique could not be successfully replicated using cultivated cotton as a plant tissue source.

Davidonis and Hamilton were the first to report successful regeneration of whole plants from somatic embryos of cotton. Davidonis, G.H. and R.H. Hamilton, *Plant Sci. Lett.* 32:89-93 (1984). These experimentors used immature cotyledon tissues of the cultivar Coker 310. The basic medium used consisted of Linsmaier and Skoog (LS) salts, vitamins, and the phytohormones NAA and kinetin. Linsmaier, E.M. and F. Skoog, *Physiol. Plant.* 18:100-127 (1965). The tissues used in this report had been used in culture for several years without reports of similar results, and the exact procedures necessary to replicate this process are still not widely known.

During this same year, the regeneration of plants from several different California cotton cultivars was reported, with a protocol sufficiently developed that it could be replicated by the authors in reasonable time periods. Rangan, T.S. et al., *In Vitro* 20:256 (1984). In their procedure, several tissues, like cotyledons, immature embryos, and hypocotyl tissue, were cultured on Murashige and Skoog (MS) medium (Murashige, T. and F. Skoog, *Physiol. Plant.* 15:473-497, 1962) plus the phytohormones NAA and kinetin. After three-to-four months of culture, these tissues gave rise to embryogenic callus and somatic embryos. The embryos were then transferred to a low salt medium, e.g., Beasley and Ting's (BT) medium (Beasley, C.A. and I.P. Ting, *Amer. J. Bot.* 60:130-139, 1973) plus casein hydrolysate, which permitted some of them to germinate and grow into whole plants. About 200 plants were recovered using the Acala SJ-5 cultivar. Some sterility in the plants was observed and only 20% of the plants showed somaclonal variation.

Somatic embryogenesis was observed using the Coker line 312 and a Texas race stock called T25. Robacker, D.C. and T.W. Zimmerman, *In the Ann. Mtg. of the American Society of Agronomy*, November 25-30, Las Vegas, NV. P. 85 (1984). The basal medium consisted of MS salts, the vitamins inositol and thiamine, sucrose, and the phytohormones NAA, 2,4 dichlorophenoxyacetic acid (2,4-D) and kinetin. Hypocotyls were used as the original tissue source. Even though embryos were recovered and cultured onto BT medium, no plants were recovered.

Other investigators have also reported somatic embryogenesis and plant regeneration. (Trolinder, N.L. and J.R. Goodin, *In the Proc. of the Beltwide Cotton Production Research Conferences*, January 6-11, 1985, New Orleans, LA. P. 46; and Mitten, D.H. *In the Proc. of the Beltwide Cotton Production Research Conferences*, January 6-11, 1985, New Orleans, LA. P. 57-58). These procedures were not published in detail, but based on the presentations, enough data was collected such that certain themes began to emerge. One investigator achieved somatic embryogenesis using the Coker 310 cultivar, immature embryos, and hypocotyl tissues on

MS medium plus the phytohormones NAA and 2iP (or kinetin). While clear evidence was shown for somatic embryogenesis, the recovery of whole plants from these cultures was less clear. Other experimenters provided a clear and concise protocol. Repeatability was shown as was the recovery of intact plants. While they were able to obtain somatic embryos from several different lines, the best cultivars belonged to the Coker pedigreed lines 5110 and 312 and one Texas race stock, T25. Other cultivars were unable to complete the regeneration process, i.e. convert to whole plants, or were unable to form mature somatic embryos. Basically, their protocol used MS medium, B5 vitamins, and the phytohormones 2,4-D and kinetin.

These investigators have been focusing on plant regeneration of somatic non-transformed cotton tissues, but strategies directed toward the genetic engineering plant lines typically generally involve two complementary processes. The first process involves the genetic transformation of one or more plant cells of a specifically characterized type. By transformation it is meant that a foreign gene, typically a chimeric gene construct, is introduced into the genome of the individual plant cells, typically through the aid of a vector which has the ability to transfer the gene of interest into the genome of the plant cells in culture. The second process then involves the regeneration of the transformed plant cells into whole sexually competent plants. Neither the transformation nor regeneration process need be 100% successful, but must have a reasonable degree of reliability and reproducibility so that a reasonable percentage of the cells can be transformed and regenerated into whole plants.

The two processes, transformation and regeneration, must be complementary. It is possible to transform certain tissues or cell types which cannot be regenerated, and it is also possible to regenerate plant tissues of a number of different tissue and cell types which have not yet been successfully transformed, as demonstrated by the investigators discussed above. The complementarity of the two processes must be such that the tissues which are successfully genetically transformed by the transformation process must be of a type and character, and must be in sufficient health, competency and vitality, so that they can be successfully regenerated into whole plants.

Successful transformation and regeneration techniques have been demonstrated in the prior art for other plant species. For example, in Barton et al., "Regeneration of Intact Tobacco Plants Containing Full-Length Copies of Genetically Engineered T-DNA, and Transmission of DNA to R 1 Progeny", Cell 32:1033 (April 1983), the transformation and regeneration of tobacco plants was reported. Similar results have been achieved in some other plant species, though not cotton.

The most common methodology used for the transformation of cells of dicot plant species involves the use of the plant pathogen *Agrobacterium tumefaciens*. *A. tumefaciens* harbors a plasmid, referred to as the tumor-inducing or Ti plasmid, which has the natural ability to transfer a segment of itself, referred to as the T-DNA (transfer-DNA), into the genome of infected plant cells. Wild-type *A. tumefaciens* use this ability to genetically transform infected cells of plants so that the plant cells become tumorous, and also synthesize one of a series of compounds, known as opines, which can be metabolized by the infecting *A. tumefaciens*. It has been found by several investigators that by removing the bulk of the T-DNA from a Ti plasmid harbored by *A. tumefaciens*, and by replacing that T-DNA with a foreign gene construction, that the *Agrobacterium* can transform infected plant cells with the foreign gene in such a fashion that the resultant cells are not tumorous, as plant cells infected with wild-type normally *A. tumefaciens* are. The foreign gene construction is then included in the cells of a whole plant regenerated from the transformed cells and is then inherited in a simple Mendelian manner. The construction can thus be treated as any inheritable trait for crop breeding purposes.

Although the regeneration of whole plants from somatic embryos of cotton has been previously reported, it has not been heretofore thought to have been possible to genetically form whole cotton plants utilizing the transformation/regeneration techniques.

It is therefore an object of the present invention to genetically engineer whole intact cotton plants and lines.

It is further an object of the present invention to genetically engineer the production of whole cotton plants utilizing transformation/regeneration techniques.

It is still further an object of the present invention to genetically engineer the production of whole cotton plants, using the *Agrobacterium* method of gene transformation followed by a reproducible regeneration technique.

These objects and others are fulfilled by the present invention as described below.

Summary of the Invention

The present invention involves the transformation of cotton cells in tissue culture and the regeneration of those cells into whole sexually mature cotton plants. It has been found that tissues derived from hypocotyl pieces of immature cotton plants can be inoculated with a culture of *A. tumefaciens* without adversely affecting the ability to elicit regenerable somatic embryo generation. Through the use of selectable markers, it is possible to screen, in the tissue culture stage, for transformed clonal growth of regenerable tissues to obtain whole plant development.

In brief, the process involves the steps of inoculating (or infecting) the hypocotyl pieces of immature cotton plants with an *A. tumefaciens* strain; tissue culture plating of the infected tissues; removing the bacteria from the infected tissues and simultaneously selecting tissues resistant to a selectable marker; amplifying the resistant tissues; and regenerating into whole plants.

The present invention is thus useful for the general genetic engineering of cotton plants and lines through the insertion into the germ line of such plants selected foreign genes.

Description of the Figures

Figure 1 is a schematic restriction enzyme mapping illustration of plant carrier plasmid pCMC92.

Fig. 2 is a restriction enzyme mapping illustration of the plant transformation plasmid pCMC1204.

Detailed Description of the Invention

The method of genetically engineering cotton plants and lines according to the present invention can, as stated above, be broadly thought of as involving two related processes. The first process is the genetic transformation of cotton cells or tissues in tissue culture. The second broad process is the regeneration of intact, sexually mature cotton plants from genetically transformed cells. The two processes are interdependent in that the tissues which are transformed in the regeneration process must be of a kind and of a competence so that they can be regenerated through the appropriate regeneration process. The present process envisions the genetic transformation of tissues in culture derived from hypocotyl explants excised from cotton plants four to six days after germination. The transformed tissues derived from the hypocotyl pieces can be induced to form embryonic structures, referred to here as somatic embryos, which can be regenerated into whole, relatively normal, and sexually competent cotton plants.

The transformation technique of the present invention is one which makes use of the Ti plasmid of *A. tumefaciens*. In using an *A. tumefaciens* culture as a transformation vehicle, it is most advantageous to use a non-oncogenic strain of the *Agrobacterium* as the vector carrier so that normal non-oncogenic differentiation of the transformed tissues is possible. It is also preferred, as a matter of convenience in preparing the actual genetic vector for transfer into the plant cells, that the *Agrobacterium* harbor a binary Ti plasmid system. In such a binary system, the *Agrobacterium* carries a first Ti plasmid having a virulence region therein and a second, chimeric plasmid, which contains therein the border regions of the T-DNA region of a wild-type Ti plasmid surrounding a chimeric gene construction including a foreign gene of interest. Binary Ti plasmid systems have been shown effective to transform plant cells. De Framond, *Biotechnology*, 1:262-269 (1983); Hoekema et al., *Nature*, 303:179 (1983). Such a binary system is preferred because the smaller plasmid; containing the T-DNA borders, can be constructed and manipulated most easily in an alternative host, such as *E. coli*, and then transferred into *Agrobacterium*. To be effective once introduced into cotton cells, the chimeric construction including foreign gene of interest must, in addition to a heterologous gene coding for a product of interest, contain a promoter which is effective in cotton cells to cause transcription of the gene of interest and a polyadenylation sequence or transcription control sequence also recognized in cotton cells. Promoters known to be effective in cotton cells include the nopaline synthase promoter, isolated from the T-DNA of *Agrobacterium*, and the cauliflower mosaic virus 35s promoter. It is also preferred that the binary plasmid harboring the foreign gene of interest also contained therein one or more selectable marker genes so that the transformed cells can be selected from non-transformed cells in culture. Preferred marker genes include antibiotic resistance genes so that the appropriate antibiotic can be used to segregate and select for transformed cells from among cells which are not transformed.

The heterologous gene selected for insertion into the cotton tissues can be any foreign gene selected to achieve a useful result if expressed in cotton plants. For example, expression of the *Bacillus thuringiensis* crystal protein toxin in the cells of cotton plants would make those cells toxic if ingested by *Lepidoptera* insects, thus imbuing the plants with a useful pest resistance. Expression of a viral coat protein in the cotton plant cells would make the plant resistant to infection by that virus. Transcription of negative strand RNA's in cotton plant cells can be useful for inhibition of undesirable endogenous genes or for disease resistance traits. As is understood by those skilled in the art, the demonstration here of the expression of a foreign protein in a cotton plant can readily be repeated with any other foreign gene of interest.

To commence a transformation process in accordance with the present invention, it is first necessary to construct the foreign genes of interest and properly introduce them into a transformation competent, but non-oncogenic, strain of *Agrobacterium tumefaciens*. The details of the construction of the vectors containing such foreign genes of interest are known to those skilled in the art of plant genetic engineering and do not differ in kind from those practices which have previously been demonstrated to be effective in tobacco, petunia, and other model plant species. The foreign gene should obviously be selected to accomplish some desirable effect in cotton plant cells. This effect may be growth promotion, disease resistance, a change in plant morphology or plant product (i.e. lint) quality, or any other change which can be accomplished by genetic manipulation. The chimeric gene construction can code for the expression of one or more exogenous proteins, or can cause the transcription of negative strand RNAs to control or inhibit either a disease process or an undesirable endogenous plant function.

To initiate the transformation and regeneration process for cotton plants, it is necessary to first surface sterilize cotton seeds to prevent inadvertent contamination of the resulting culture. The seeds are then allowed to germinate on an appropriate germinating medium containing a fungicide.

Four to six days after germination the hypocotyl portion of the immature plant is removed and sectioned into small segments averaging approximately 0.5 centimeters apiece. The hypocotyl explants are allowed to stabilize and remain viable in a liquid or agar plant tissue culture medium.

Once the hypocotyl segments have stabilized, they can promptly be inoculated with a suspension culture of transformation competent non-oncogenic *Agrobacterium*. The inoculation process is allowed to proceed for three to five days at room temperatures, i.e., 24° C.

At the end of the inoculation time period, it is necessary first to rinse off the excess *Agrobacterium*. Then the remaining treated tissues can be transferred to a second agar medium, which also contains one or more antibiotics toxic to *Agrobacterium*, but not to hypocotyl tissues, at a concentration sufficient to kill any *Agrobacterium* remaining in the culture. Suitable antibiotics for use in such a medium include carbenicillin and cefotaxime. The tissues are then given a period of from one to ten days to recover from the transformation process and are then continued in culture. 5

The tissues are now cultivated on a tissue culture medium which, in addition to its normal components, contains a selection agent, the selection agent being one toxic to non-transformed cotton cells but not to transformed cotton cells which have incorporated genetic resistance to the selection agent and are expressing that resistance. A suitable tissue culture medium is the MS medium to which is added the phytohormones 2,4 dichlorophenoxy-acetic acid (2-4,D), 6-furfurylaminopurine and a gelling agent. Suitable selection agents include both antibiotics and herbicides. Suitable antibiotic traits which may serve as dominant selectable markers include the aminoglycoside phosphotransferase-3'-II (APH-(3')-II) gene, also referred to as the neomycin phosphotransferase II gene (NPTII), which code for resistance to the antibiotic kanamycin, and the APH-(3')-IV gene which codes for resistance to Hygromycin B. Kanamycin, G418 and Hygromycin B are aminoglycosides that will stop the growth of non-transformed cotton cells, but these antibiotics are phosphorylated by the appropriate enzyme if it is expressed in the transformed cells. Another suitable antibiotic selection agent is Chloramphenicol which can be acetylated and rendered non-toxic by the enzyme produced by the CAT (Chloramphenicol acetyl transferase) gene. Antibiotic dosed media allows only transformed cells to continue to grow and thrive. Thus the transformed cells, or calli, are allowed to grow on the selective medium. The surviving transformed tissues are transferred to a secondary medium to induce somatic embryogenesis. The surviving transformed tissue will thus continue to form into somatic embryos, which can then be regenerated through the regeneration technique of the present invention or through any other alternative plant regeneration protocols which use cotton somatic embryos as their starting point. 10 15 20

The selection process should continue for an extended time, i.e. 3-4 months, because of the slow growth of even transformed tissues on the antibiotic medium. Subcultures are made every 4-6 weeks to replenish nutrients and antibiotics. As the transformed cells are selected and amplified, individually derived cell lines are identifiable and can be removed and separately amplified. 25

The regeneration technique in accordance with the present invention begins with the tissues resulting from the transformation process. These tissues are putatively transformed calli which can generate somatic embryos when cultivated on appropriate embryo induction media. One technique for regenerating these somatic embryos to whole plants is disclosed here, but it is to be understood that other techniques are also possible, once transformed embryogenic tissues are produced. 30

The regeneration technique used by the applicants here thus begins with the tissues resulting from the transformation process. The cotton tissue calli, generated from the hypocotyl segments of the cotton plants, and putatively transformed, are placed onto somatic embryo induction media directly. At this point, the antibiotic selection agent should be removed from the culture medium, but otherwise the medium may remain constant. These calli, cultured on the somatic embryo induction medium, will form small embryoidal structures, which have been termed somatic embryos. It may take as long as two to three months for the somatic embryos to emerge and mature. Approximately 5 to as many as 20 somatic embryos will emerge from a single callus in an agar formulation of a somatic embryo induction medium. Many of the somatic embryos thus produced will be regenerable into whole plants in accordance with the technique described here. 35 40

When the developing somatic embryos are large enough, i.e. to a size of 4 mm or more in length, and if they appeared to have good embryonic development, i.e. usually having a cotyledon and a radicle, they may be transferred to large test tubes and hosted on fine vermiculite. The vermiculite is saturated with Stewart and Hsu (SH) medium (Planta 137:113 (1977)) plus the phytohormones indole acetic acid, 6-furfurylaminopurine and gibberellic acid. Small plantlets, having two to three leaves, eventually develop. 45

Once plantlet growth is established, i.e., the 2-3 leaf stage, the plants can now move into plant pots with vermiculite soil. They may be watered and fertilized as needed. They may also need to be hardened off, before greenhouse exposure. The plantlets may be repotted when they have 4-6 leaves after which they will continue to grow until mature. 50

The following examples will serve to illustrate the process of the invention without limiting the same.

EXAMPLE 1

Transformation of Callus

This example was designed to illustrate the transformation of cotton tissues in culture using virulent and avirulent strains of *A. tumefaciens* which carried plasmids that encoded for the marker enzymes neomycin phosphotransferase II (NPTII) (also known as APH-3'-II) and/or chloramphenicol acetyl transferase (CAT). The callus tissues were amplified over a period of several months on a selection medium. Tissue samples were removed and assayed for expression of the enzymes and the remaining tissues were used for DNA analysis. The details of the example are as follows: 55 60

Seeds of cultivated cotton (*Gossypium hirsutum* L.) of Coker lines 310, 312, 5110 Deltapine 61, and Stoneville 213 were surface sterilized with 3% sodium hypochlorite for twenty minutes. The seeds were then rinsed three times with sterile distilled water plus cefotaxime (500 mg/l). The seeds were allowed to germinate 65

in SH medium containing the fungicide benomyl (50 mg/l). Four to six days after germination, hypocotyl explants were removed, cut into 0.5 cm pieces, and placed on a support medium containing agar (0.8%) and water.

The hypocotyl pieces were then inoculated with diluted (1:10) overnight culture of *A. tumefaciens*. The suspension culture of *A. tumefaciens* contained approximately 10^8 bacteria per milliliter.

The particular *A. tumefaciens* strain used harbored a binary Ti plasmid system containing one Ti plasmid carrying the so-called virulence region and a second Ti plasmid containing T-DNA border regions from a virulent Ti plasmid (pCMC91, pCMC92 or pCMC1204) and also containing an antibiotic resistance gene (NPT II). One plasmid (pCMC1204) also carried a foreign gene (CAT) between the T-DNA borders. The infection of the *A. tumefaciens* on the immature embryonic tissues was allowed to proceed for three to five days at room temperature.

After incubation at room temperature and lighting, the tissues were transferred to Murashige and Skoog salts with B5 vitamins (Gamborg et al., *Exp. Cell Res.* 50:151-158 (1968)) plus antibiotics, the phytohormones 2,4-D (0.1 mg/l) and 6-furfurylaminopurine (0.1 mg/l), and the gelling agent Gelrite (1.6 g/l) (Gelrite is a registered trademark of Kelco Company, San Diego, California) plus magnesium chloride (750 mg/l). The antibiotics used to kill the bacteria were cefotaxime (50-100 mg/l) and carbenicillin (400-500 mg/l). Kanamycin sulfate (5-50 mg/l) was included in the medium and used as a selective agent. Subcultures were made every four to six weeks to replenish depleted nutrients and antibiotics.

After three to four months, individually derived cell lines were labelled and maintained on a selection medium for tissue amplification. Tissues were incubated at 30° C with a 16 hour photoperiod (50-100 $\mu\text{mol}/\text{m}^2/\text{s}$).

NPTII assays were carried out using crude cell extracts prepared from calli or plant tissues preincubated with a buffer solution (30 mM NaCl, 15 mM NH_2Cl , 3 mM MgCl_2 , 5.5 mM Na EDTA, 2.3 mM Tris, 0.2 mM dithiothreitol (DTT), 105 mM sucrose at a pH of 7). Following three freeze-thaw cycles in liquid nitrogen, tissues were ground in buffer containing 37 mM Na EDTA, 126 mM NaCl, 83 mM NH_2Cl , 7.7 mM Tris, and 15 mM DTT. To protect against protease degradation of NPTII, 2.5 mM phenylmethylsulfonyl fluoride and 0.6 mg/ml leupeptin, 1.05 mg/ml soybean trypsin inhibitor and 5.0 mg/ml bovine serum albumin were added to the buffer. Forty microliters of extraction buffer was used per 100 mg (fresh weight) of callus or plant tissue. Supernatants were collected following centrifugation. Electrophoretic analysis of NPTII activity was carried out according to Paszkowski, J. et al., *EMBO. J.* 3:2717-2722 (1984); and Reiss, B. et al., 1984, *Gene*, 30:211-218 (1984).

Plant tissues were extracted according to the procedure of Herrera-Estrella et al., *Nature*, 303:209-213 (1983). The extracted plant tissues were CAT assayed as described by Gorman, C.M. et al., *Molec. Cell. Biol.* 2:1044-1051 (1982).

Table 1 lists the various vectors that were tested, the number of transformed lines obtained and the result of their NPTII test. Table 2 describes data collected for samples of two of the vectors.

Table 1

Vector constructions that have been tested in cotton

Vector	No. of Trans- formed Lines Recovered	No. of Lines Tested for NPTII	No. of Lines With (+) Assays	%
A6	4	4	0	0
A6(pCMC91)	21	21	16	76
A6(pCMC92)	16	16	16	100
LBA4404(pCMC91)	17	17	15	88
LBA4404(pCMC92)	4	4	4	100
LBA4404(pCMC1204)	40	6	5	83

The A6 construction does not carry the Kanamycin resistance gene and, therefore, will not produce the NPTII protein. A6 is a virulent *Agrobacterium* strain. LBA4404 is a non-virulent strain. Plasmids pCMC91 and pCMC92 carry the NPT II gene alone between the T-DNA border segments. Plasmid pCMC1204 carries the NPT II and CAT genes in tandem.

Table 2
NPTII activity in transformed cotton callus

<u>Vector/Sample</u>	<u>cpm/20 ul</u> <u>crude extract</u>	<u>ng enzyme/20 ul *</u>
<u>Controls:</u>		
Stoneville 231	102	L 0.1
Coker 310	0	L 0.1
Deltapine 61	25	L 0.1
<u>Vector LBA4404(92)</u>		
1	26,320	3.06
2	77,016	8.97
3	31,190	3.63
4	53,413	6.20

Nanogram values were calculated from counts obtained from a known amount of NPTII enzyme run on the same gel.

"L" indicates less than.

Nanogram values were calculated from counts obtained from a known amount of NPTII enzyme run on the same gel. "L" indicates less than.

The results indicate the expression of at least one enzyme (NPTII) and/or in most cases the expression of both enzymes (NPTII and CAT) when they were incorporated together on the same plasmid in tissues of cotton callus in culture.

EXAMPLE 2

Transformation and Regeneration

In this example, cotton tissues were transformed using an avirulent strain of *A. tumefaciens* (LBA4404) which carried a plasmid (pCMC1204) that encoded for the marker enzyme NPTII and also for CAT. Over the next several months callus tissues were amplified on selection medium. Tissue samples were removed and assayed for expression of the enzymes. The remaining callus tissues were used to regenerate plants. Details of the example follow.

The plasmid pCMC1204 was constructed from components of a bipartite vector system similar to those previously described in the art. de Framond et al., *Bio/technology*, 1:262-269 (1983), Bevan, *Nuc. Acids Res.*, 12:8711-8721 (1984). An expression cassette for the CAT gene was prepared by ligating the Hind III/Sau 3AI CAT coding fragment from pSV2CAT (Gorman et al. *Mol. Cell. Biol.* 2:1044-1051 (1982)) into the Hind III and Bam HI sites of pCMC66. The plasmid pCMC66 is an expression plasmid which employs the promoter and polyadenylation regions from the nopaline synthase *nos* gene of pTiT37 of *Agrobacterium*. The resulting CAT expression plasmid, designated pCMC1201, was linearized with Sal I which cuts the plasmid once immediately downstream of the polyadenylation region, and was inserted into the unique Sal I site on the carrier plasmid pCMC92. The carrier plasmid pCMC92 is a wide-host range replicon carrying the T-DNA border regions from *Agrobacterium* and a chimeric NPTII gene. The chimeric NPTII gene is formed from the nopaline synthase promoter and polydenylation sequence from pTiT37, i.e. the same control sequences as in pCMC66, and the NPTII coding region from Ti5. The product of the ligation was designated pCMC1204. Plasmid pCMC1204 was mobilized from *E. Coli* into *A. tumefaciens* strain LBA4404 in a triparental mating with a helper strain of *E. Coli* carrying pRK2013. Transconjugants were selected on plates containing streptomycin, sulfadiazone and carbenicillin at 100 microgram/ml. Single colonies were picked for amplification for inoculation of plant tissues.

The construction and use of both plasmids pCMC66 and pCMC92 is described in more detail in published patent application WO 85/04899 by Barton and Gelfand. The plasmid pCMC92 is on deposit with the ATCC, accession number 53093 and not only contains the chimeric NPTII gene and T-DNA borders, but contains the nopaline synthase promoter and polyadenylation sequences which can be removed from that plasmid and used with any desired heterologous coding sequence.

Hypocotyl pieces were inoculated with diluted (1:10) overnight cultures of *A. tumefaciens*. After three days incubation at room temperature (23° - 27° C) and lighting, the tissues were transferred to MS salts with B5 vitamins plus antibiotics, the phytohormones 2,4-D (0.1 mg/l) and 6 furfurylaminopurine (0.1 mg/l) and the

gelling agent Gelrite (1.6 g/l), plus magnesium chloride (750 mg/l). The antibiotics used to kill the bacteria were cefotaxime (50-100 mg/l) and carbenicillin (400-500 mg/l). Kanamycin sulfate (5-50 mg/l) was included in the medium and used as a selective agent. Subcultures were made every four to six weeks to replenish depleted nutrients and antibiotics. After three to four months, individually derived cell lines were labeled and maintained on the selection medium for tissue amplification. The tissues were incubated at 30° C for a 16 hour photoperiod (50-100 $\mu\text{mol}/\text{m}^2/\text{s}$).

After amplification, the antibiotics were discontinued and the transformed tissues were maintained on the same medium. After an additional 2 to 3 months, embryogenic calli emerged to give rise to somatic embryos. When the embryos were large enough, i.e., 4 mm or more in length, and appeared to have good embryo development, cotyledon and radicle present, they were transferred to large test tubes containing "fine" vermiculite. The vermiculite was saturated with SH medium plus the phytohormones indole acetic acid, 6-furfurylaminopurine and gibberellic acid, all at 0.1 mg/l. The embryos were incubated at a 30° C, 16 hour photoperiod. Upon germination, i.e., 2-3 leaf stage, plantlets were transferred to pots filled with vermiculite and watered and fertilized as needed. The plantlets were enclosed in a beaker for about one week to help harden-off the leaves. Once potted-plantlet growth was established (4-6 leaf stage), they were moved to the greenhouse. Adapted plants were repotted in a commercial soil mixture, Metro Mix 360 (Metro Mix is a registered trademark of W. R. Grace and Co.), and maintained until mature.

The NPTII assays were performed according to the process outline in Example 1. The assays indicated NPTII activity in the leaf tissue of several of the regenerated cotton plants. Some plants grown from callus testing positive for NPTII activity tested negative, however.

To confirm the presence of the plasmid in the transformed plants, a Southern blot analysis was performed. The Southern blot analysis was performed with the restriction enzymes *Ava* I and *Hind* III, corresponding to the NPTII coding region. The plant DNA, extracted and digested, was hybridized with a radiolabeled negative strand NPTII sequence. A hybridizing band indicated presence of the expected fragment in the plant tissues in at least one copy number.

The plasmid pCMC 1204 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA, on December 3, 1986 under ATCC accession No. 67275.

The above deposit was made pursuant to a contract between the ATCC and the Cetus Corporation, a partner in the assignee of the present invention. The contract with the ATCC provides for permanent availability of the progeny of these cell lines to the public on the issuance of the US patent describing and identifying the deposit or the publication or laying open to the public of any US or foreign patent application, whichever comes first, and for availability of the progeny of these cell lines to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC Section 122 and the Commissioner's rules pursuant thereto (including 37 CFR Section 1.14 with particular reference to 886 O.G. 638). The assignee of the present application has agreed that if the cell lines on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable culture of the same cell line.

The present invention is not to be limited in scope by the microorganisms deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any microorganisms which are functionally equivalent are within the scope of this invention. Indeed various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

In one embodiment a method for introducing genes into cotton plants and plant lines, comprises the following steps in sequence:

- a) surface sterilizing cotton seeds;
- b) allowing said cotton seeds to germinate thus forming immature cotton plants, said immature cotton plants including hypocotyl tissue;
- c) exposing said hypocotyl tissue to a culture of transformation competent non-oncogenic *Agrobacterium tumefaciens* harboring a Ti plasmid having a T-DNA region including both a foreign chimeric gene and a selection agent resistance gene;
- d) culturing said hypocotyl tissue on a medium containing at least one antibiotic toxic to said *Agrobacterium tumefaciens* but not toxic to cotton cells;
- e) culturing said tissue of step d) in the presence of a selection agent for which the resistance gene encodes for resistance so as to select for plant cells transformed with the T-DNA region;
- f) inducing somatic embryo formation in the exposed tissue in culture; and
- g) regenerating the somatic embryos into whole cotton plants.

Claims

1. A method of introducing genes into cotton plants and plant lines characterised in that it comprises exposing hypocotyl tissue of immature cotton plants to a culture of transformation competent non-oncogenic *Agrobacterium tumefaciens* harboring a Ti plasmid having a T-DNA region including both a foreign chimeric gene and a selection agent resistance gene.

2. A method as claimed in claim 1, characterised in that it further comprises culturing the exposed tissue in the presence of a selection agent for which the resistance gene encodes for resistance so as to select for plant cells transformed with the T-DNA region; inducing somatic embryo formation in the exposed tissue in culture; and regenerating the somatic embryos into whole cotton plants.

3. A method as claimed in either claim 1 or claim 2, characterised in that said exposing step is preceded by surface sterilization of cotton seeds followed by germination of said cotton seeds to form said immature cotton plants.

4. A method as claimed in any one of the preceding claims, characterised in that the hypocotyl tissue comprises pieces of hypocotyl explants which are removed from said immature cotton plants.

5. A method as claimed in any one of the preceding claims, characterised in that the culture of *Agrobacterium tumefaciens* harbors a binary Ti plasmid system in which a virulence trait is carried on a plasmid separate from the plasmid carrying the T-DNA region.

6. A method as claimed in claim 5, characterised in that the T-DNA region includes only the T-DNA right and left borders from the T-DNA of a wild-type Ti plasmid.

7. A method as claimed in any one of claims 2 to 6, characterised in that the selection agent is an antibiotic and the resistance gene codes for antibiotic resistance.

8. A method as claimed in claim 7, characterised in that the antibiotic resistance gene is the NPTII gene and the antibiotic is selected from the group consisting of Kanamycin and G418.

9. A method as claimed in claim 7, characterised in that the antibiotic resistance gene is the CAT gene and the antibiotic is Chloramphenicol.

10. A method as claimed in claim 7, characterised in that two antibiotics and two antibiotic resistance genes are used, said antibiotics being preferably selected from the group consisting of Hygromycin B, Chloramphenicol, and one of Kanamycin and G418.

11. A method as claimed in any one of the preceding claims, characterised in that it further includes, after the step of exposing the tissue to said *Agrobacterium tumefaciens*, a step of culturing the tissue on a medium containing at least one antibiotic toxic to said *Agrobacterium tumefaciens* but not toxic to cotton cells.

12. A method as claimed in any one of claims 2 to 11, characterised in that the step of inducing embryo formation includes culturing the plant tissue on a culture media containing at least one auxin or cytokinin.

13. Cotton plants characterised in that they are produced by a method as claimed in any one of the preceding claims.

14. Cotton somatic embryos characterised in that they are produced by a method as claimed in any one of claims 1 to 12.

15. Cotton seeds characterised in that they are produced by plants as claimed in claim 13.

16. Cotton seeds capable of germination into cotton plants comprising in their genome a chimeric gene construction including a foreign gene and promoter and control sequences operable in plant cells, the chimeric gene construction being effective in the cells of the cotton plant to express a cellular product coded by the foreign gene.

17. Cotton seeds as claimed in claim 16, characterised in that the cellular product is an exogenous protein or an RNA capable of producing a somatic change to the cotton plant.

18. Cotton seeds as claimed in claim 16, characterised in that the foreign gene codes for the production of a negative RNA strand effective to condition a somatic change in the cotton plant grown from the seed.

19. Cotton seeds as claimed in any one of claims 16 to 18, characterised in that the promoter sequence is the nopaline synthase promoter from *Agrobacterium tumefaciens* or the cauliflower mosaic virus 35s promoter.

20. Cotton plants characterised in that they are germinated from seeds as claimed in any one of claims 16 to 19.

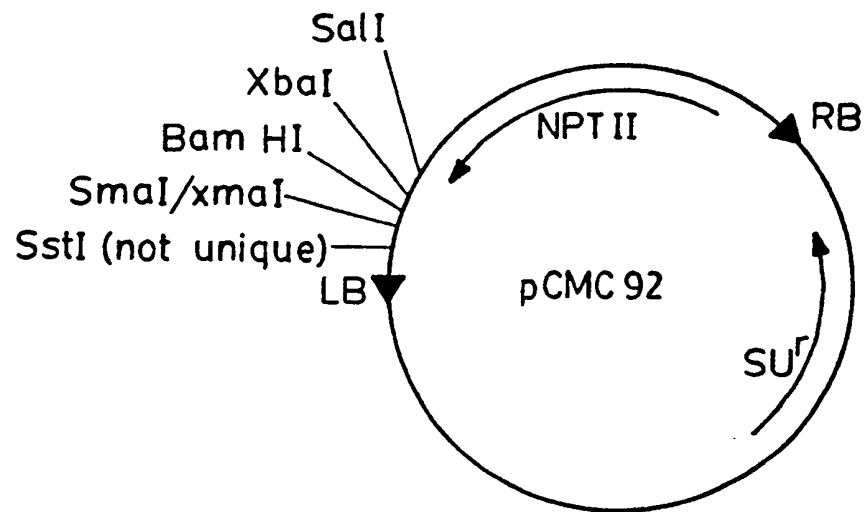


FIG 1

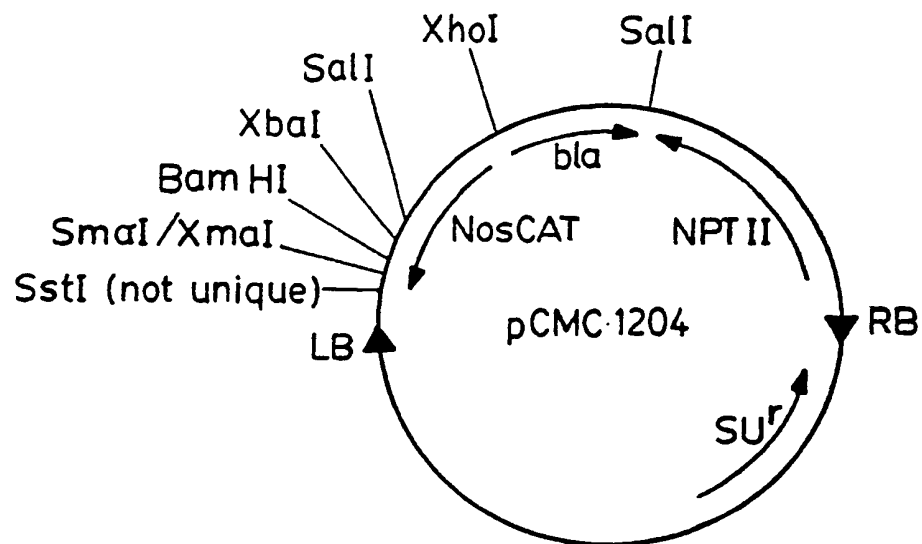


FIG. 2

12

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54 **Genetic engineering of cotton plants and lines.**

57 A method is disclosed to achieve genetic transformation of cotton plants and lines. Immature cotton tissues are genetically transformed in vitro, by Agrobacterium-mediated genetic transformation. The resultant cotton tissues are subjected to a selection agent or agents to screen for transformants. The transformed cultures are then induced to commence somatic embryogenesis. One possible regime for regenerating such somatic embryos into whole cotton plants is disclosed.

EP 0 270 355 A3



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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X	METHODS IN ENZYMOLOGY, vol. 101, 1983, pages 433-481, Academic Press, Inc.; G.-Y. ZHOU: "Introduction of exogenous DNA into cotton Embryos" * Page 440, paragraph 1 * ---	16, 17, 20	C 12 N 15/00 A 01 G 7/00 A 01 H 1/00 // C 12 P 21/02
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Y	PLANT CELL REPORTS, vol. 5, 25th April 1986, pages 81-84, Springer-Verlag; S. McCORMICK et al.: "Leaf disc transformation of cultivated tomato (L. esculentum) using Agrobacterium tumefaciens" * Abstract * ---	1-15, 19	TECHNICAL FIELDS SEARCHED (Int. Cl. 4) C 12 N A 01 G C 12 P
Y	PLANT CELL REPORTS, vol. 5, no. 3, 1986, pages 178-181; R.C. SHOEMAKER et al.: "Characterization of somatic embryogenesis and plant regeneration in cotton (Gossypium hirsutum L.)" * Whole document, particularly "Materials and methods" * --- -/-	2, 12	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 27-03-1990	Examiner MADDOX A.D.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
P,X	BIOTECHNOLOGY, vol. 5, no. 3, March 1987, pages 263-266, New York, NY, US; P. UMBECK et al.: "Genetically transformed cotton (<i>Gossypium hirsutum</i> L.) plants" * Whole document *	1-20	
A	IN VITRO, vol. 20, 1984, page 256; T.S. RANGAN et al.: "Somatic embryogenesis in tissue cultures of <i>Gossypium hirsutum</i> L." * Abstract point 65 *	2,12	
A	THEOR. APPL. GENETICS, vol. 73, 1986, pages 11-15; A.J. TRULSON et al.: "Transformation of cucumber (<i>Cucumis sativus</i> L.) plants with <i>Agrobacterium rhizogenes</i> " * Page 12, left-hand column "Plant regeneration" *	2,12	
A	WO-A-8 504 899 (AGRACETUS) * Page 35, lines 25-32; page 47 *	9	
A	NUCLEIC ACIDS RESEARCH, vol. 13, no. 19, October 1985, pages 6981-6988, IRL PRESS LTD, Oxford, GB; J. VELTEN et al.: "Selection-expression plasmid vectors for use in genetic transformation of higher plants" * Abstract *	9	
A	GENETIC ENGINEERING NEWS, vol. 5, no. 4, April 1985, page 3; F. GEBHART: "Calgene expresses recombinant gene for herbicide tolerance" * Left-hand column, paragraphs 5,6 *	1-20	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 27-03-1990	Examiner MADDOX A.D.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 27-03-1990	Examiner MADDOX A. D.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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